Effects of in ovo injection with selenium on immune and antioxidant responses during experimental necrotic enteritis in broiler chickens[1]

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ABSTRACT This study was conducted to investigate the effects of in ovo injection of Se on modulating the immune system and antioxidant responses in broiler chickens with experimental necrotic enteritis. Broiler eggs were injected at 18 d of embryo age with either 100 μL of PBS alone or sodium selenite (Na_2SeO_3) in PBS, providing 0 (SS0), 10 (SS10), or 20 (SS20) μg of Se/egg. At 14 d posthatch, PBS-treated and uninfected chickens were kept as the control group, whereas the remaining chickens were orally infected with 1.0×10^4 sporulated oocysts of *Eimeria maxima* (SS0, SS10, SS20). At 18 d posthatch, *E. maxima*-infected chickens were orally infected with 1.0×10^9 cfu of *Clostridium perfringens*. Infected control SS0 group showed significantly decreased BW compared with the uninfected control. However, SS20 group showed significantly increased BW compared with the infected control SS0 group, whereas the BW were similar among uninfected control and infected SS10 and SS20 groups. The SS10 group showed significantly lower intestinal lesions compared with the SS0 group, and oocyst production was decreased in both SS10 and SS20 groups. Serum malondialdehyde level and catalase activity were also decreased in both SS10 and SS20 groups, whereas the superoxide dismutase level was significantly lower in the SS10 group compared with the SS0 group. The SS20 group showed significantly higher levels of transcripts for IL-1β and IL-6 in intestine, and SS10 and SS20 groups had higher levels of transcripts for IL-8 and inducible nitric oxide synthase expression and decreased glutathione peroxidase 7 mRNA levels compared with the SS0 group. The SS10 and SS20 groups also showed increased serum antibody levels to *C. perfringens* α-toxin and NetB toxin compared with the SS0 group. These collective results suggest that the injection of Se into the amniotic cavity of developing eggs may be beneficial for enhancing immune and antioxidant responses in the hatched chickens exposed to the necrotic enteritis-causing pathogens.

Key words: selenium, chicken, antioxidant, immunology, necrotic enteritis

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INTRODUCTION

Selenium is a nonmetallic essential micronutrient that regulates major physiologic pathways of humans and animals, including those of the immune and antioxidant systems. Selenium deficiency increases susceptibility to infectious diseases and enhances biochemical stress in response to pathogen colonization (Wang et al., 2009; Smith et al., 2011). Selenium-deficient mice showed increased susceptibility to infection by *Listeria monocytogenes* concomitant with reduced antioxidant enzyme activities and decreased innate immune responses (Wang et al., 2009).

Necrotic enteritis (**NE**) and avian coccidiosis are among the most important diseases in commercial poultry production, both inflicting substantial economic losses worldwide (Van der Sluis, 2000; Timbermont et al., 2011; Shirley and Lillehoj, 2012). It has been reported that a host mucogenic response to an intestinal coccidial infection promotes the onset of NE (Collier et al., 2008; Lee et al., 2011, 2013; Jang et al., 2013). Traditionally, field outbreaks of NE have been controlled prophylactically by in-feed antibiotics

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(Lovland and Kaldhusdal, 2001; Van Immerseel et al., 2009; Lee et al., 2011). However, the global incidence of *Clostridium perfringens*-associated NE has substantially increased in the last decade, primarily due to the decreased use of in-feed antibiotics as growth promoters. Necrotic enteritis costs the international poultry industry approximately 2 billion US dollars annually due to expenses associated with antimicrobial drug use and impaired BW gain (Lee et al., 2011). For these reasons and because of the potential for the emergence of drug-resistant pathogens, recent interest has focused on developing drug-free disease strategies to control NE and to reduce the risk factors associated with NE (Lee et al., 2008, 2011, 2013). Vaccination with attenuated strains of *C. perfringens* or recombinant vaccines, and dietary supplementation with phytonutrients or micronutrients offer 2 such alternative approaches (Cooper et al., 2009; Jang et al., 2012; Lee et al., 2013).

In ovo injection of broiler eggs with vaccines or dietary nutrients enhances the growth and development of the chicken embryo, promotes early gut maturation, and improves posthatch immune status (Ohta and Kidd, 2001; Tako et al., 2005; Foye et al., 2007; Lee et al., 2010). Although in ovo treatment with Se seems feasible for potentiating the protective immune mechanisms against avian NE, this approach has not been reported yet. Therefore, we hypothesized that in ovo injection of Se would enhance posthatch chicken resistance against experimental NE. To test this hypothesis, broiler eggs were injected with PBS alone or Se solutions at 18 d of embryogenesis and antioxidant and immune responses against NE were compared between the control and experimental groups.

MATERIALS AND METHODS

In Ovo Injection of Se and Environmental Conditions

Embryonated eggs of inbred broiler chickens (Moyer's Chicks Inc., Quakertown, PA) were incubated at 37.5°C with a RH of 55 to 60% for 18 d and candled to select well-developed embryos. At 18 d of embryo development, eggs were injected with 100 μL containing sterile PBS (pH 7.4) alone, or sodium selenite (Na_2SeO_3, Pancosma, Geneva, Switzerland) solutions in PBS at 10 or 20 μg of Se/egg using the Intelliject system (Avitech, Easton, MD). In this study, the Se doses were selected based on other in ovo trials reporting on immune response or antioxidant effects and low toxicity in fertilized eggs (Kury et al., 1967; Macalintal, 2012). Each egg was cleaned and positioned in a holder under an injection needle with the large end on top. With the help of a vacuum system, the disinfected needle penetrated the shell past the air cell to deliver the solutions into the amniotic cavity. After each inoculation, the needle was routinely disinfected to minimize the risk of infection. In addition, the system was designed to avoid creating negative pressure inside of the egg, thus reducing the risk of cross-contamination (Lee et al., 2010). The eggs were incubated at 36°C with a RH of 60 to 65% until hatch. At 3 d postinjection, the hatched chickens were provided with feed and water ad libitum. For each treatment (Cont, SS0, SS10, and SS20), chickens (n = 20/group) were kept in 3 brooder pens (n = 6–7 birds/pen) in an *Eimeria*-free facility for 14 d posthatch and transferred into 7 large hanging cages (n = 2–3 birds/cage) at a separate location where they were coinfected with *E. maxima* and *C. perfringens*, and kept until the end of the experimental period.

Chickens were maintained in a temperature-controlled environment at 30°C for the first 2 d of growth followed by a gradual reduction in the temperature to 23°C until end of the experiment. In general, Se-deficient basal diets are fed to chickens from hatch followed by Se treatment in ovo because a high concentration of Se diet may reduce the effects of Se supplement or the bioavailability of different Se sources in ovo (Wang et al., 2009; Habibian et al., 2013). Chickens were continuously fed from hatch with a basal diet (0.1 mg/kg of Se) ad libitum for 20 d. To facilitate the development of NE, birds were fed an antibiotic-free certified organic starter diet containing 17% CP and 61% carbohydrate between d 1 and 18 posthatch and a standard grower diet containing 24% CP and 54% carbohydrate between d 18 and 20. All diets contained a 15% Se-free mineral and vitamin mixture, 4.7% fat, and 2.4% fiber (USDA/FeedMill, Beltsville, MD). All experiments were approved by the USDA-Agricultural Research Service Institutional Animal Care and Use Committee.

NE Disease Model

A well-established NE disease model developed in our laboratory was used (Lee et al., 2011, 2013; Jang et al., 2012, 2013). Chickens in the SS0, SS10, and SS20 groups were orally infected on d 14 with *E. maxima* Beltsville strain 41A (1.0×10^4 oocysts/bird) and on d 18 with *C. perfringens* strain Del-1 (1.0×10^9 cfu/bird), a strain that we previously described and used in the NE disease model, and which expresses both α-toxin and NetB toxin (Lee et al., 2011, 2013; Jang et al., 2013).

BW, Gut Lesion Scores, and Fecal Oocyst Shedding

Body weights were measured at d 0 and 14 (before infection with *E. maxima*), and at d 20 (d 6 postinfection with *E. maxima* and d 2 postinfection with *C. perfringens*) as described (Nagy et al., 1996; Cooper et al., 2009). For gut lesions, birds (n = 5/group) were killed by cervical dislocation at d 2 postinfection with *C. perfringens*. Two equal intestinal sections of 10 cm located anterior and posterior to the diverticulum were collected and scored on a scale of 0 (none) to 4 (high)

in a blinded fashion by 3 independent observers as described (Lee et al., 2013). *Eimeria maxima* fecal oocyst numbers (n = 15/group) were determined between d 19 and 24 (d 5 to 10 postinfection with *E. maxima*) using a McMaster chamber according to the formula: total oocysts per bird = oocyst count × dilution factor × (fecal sample volume ÷ counting chamber volume) ÷ 2 (Lee et al., 2013).

Serum Antibody Levels to α-Toxin and NetB Toxin

Blood samples (5 birds/group) were collected by cardiac puncture immediately following euthanasia at d 6 postinfection with *C. perfringens* and sera were prepared by centrifugation at 1,000 × *g* for 10 min at 4°C. Ninety-six well microtiter plates were coated overnight with 1.0 μg/well of purified recombinant α-toxin or NetB toxin proteins. The plates were washed with PBS containing 0.05% Tween 20 (PBS-T) and blocked with PBS containing 1% BSA. Serum samples were diluted 1:20 and 100 μL/well was incubated with agitation for 2 h at room temperature. Following washing with PBS-T, bound antibodies were detected with horseradish peroxidase-conjugated rabbit anti-chicken IgG secondary antibody and tetramethylbenzidine substrate.

Serum Malondialdehyde Levels and Catalase and Superoxide Dismutase Activities

Blood samples (n = 5 birds/group) were collected by cardiac puncture immediately following euthanasia at d 2 postinfection with *C. perfringens* for determination of serum malondialdehyde (**MDA**) levels, and catalase (**CAT**) and superoxide dismutase (**SOD**) activities. The MDA concentration in serum was measured using the MDA Assay Kit (Life Science Specialties, Vancouver, WA). Catalase and SOD activities were quantified using the Catalase Activity Assay Kit (Cell Biolabs, San Diego, CA) and SOD Assay Kit-WST (Sigma, St. Louis, MO). Optical densities were measured at 532 nm (MDA assay), 520 nm (CAT assay), and 440 nm (SOD assay) using a UV/visible spectrophotometer (Amersham Biosciences, Buckinghamshire, UK).

Cytokine/Chemokine and Antioxidant-Related mRNA Levels in Intestine

At 2 d postinfection with *C. perfringens*, 20-cm-long jejunum tissues located proximal to Meckel's diverticulum were collected from 5 chickens per group (Lee et al., 2013). The jejunum was cut open longitudinally, gently washed with ice-cold Hank's balanced salt solution (Sigma), and the mucosal layer was carefully scraped away using a cell scraper. Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA). Five micrograms of total RNA were treated with 1.0 U of DNase I and 1.0 μL of 10 × reaction buffer (Sigma) and incubated for 15 min at room temperature. One microliter of stop solution was added to inactivate DNase I and the mixture was heated for 10 min at 70°C. The RNA was reverse-transcribed using the StrataScript first-strand synthesis system (Stratagene, La Jolla, CA) according to the manufacturer's recommendations. Real-time reverse-transcription (**qRT**) PCR oligonucleotide primers for chicken IL-1β, IL-6, IL-8, inducible nitric oxide synthase (**iNOS**), glutathione peroxidase 7 (**GPx7**), and glyceraldehyde 3-phosphate dehydrogenase (**GAPDH**) as an internal control are listed in Table 1. Amplification and detection were carried out with the Mx3000P system and Brilliant SYBR Green qPCR Master Mix (Stratagene). The reverse-transcription product was diluted to 1:10, and 5 μL was used for PCR amplification. The PCR conditions were as follows: denaturation at 95°C for 10 min followed by amplification at 72°C for 1 min for 40 cycles. Standard curves were generated using $\log_{10}$ diluted standard RNA to calculate the amplification efficiency and the levels of individual transcripts were normalized to those of GAPDH by the Q-gene program (Muller et al., 2002; Cooper et al., 2009). Each sample was analyzed in triplicate. To normalize individual replicates, the logarithmic-scaled threshold cycle (C_t) values were transformed to linear units of normalized expression before calculating means and SEM for the references and individual targets, followed by the determination of mean normalized expression using the Q-gene program.

Measurement of Se Concentrations in Tissues

Selenium concentrations of tissues and feces collected from 5 chickens per group at 6 d postinfection with *C. perfringens* were measured as described (AOAC International, 2000) using an inductively coupled plasma-mass spectrometry system (7500 series, Agilent, Santa Clara, CA). Powdered samples were dissolved by microwave digestion (MicroPrep Q2000, Questron Technologies, Mississauga, Canada) using a mixture of hydrofluoric, nitric, and perchloric acids (Merck, Whitehouse Station, NJ). Selenium concentrations were determined using external calibration curves. Mean values of measurements of unknown specimens were compared with certified quality control standards.

Statistical Analysis

A factorial experiment was done, based on a randomized complete block design with 4 replications. All data were subjected to one-way ANOVA using SPSS 15.0 for Windows (SPSS Inc., Chicago, IL). Mean values of treatment groups were compared using the Tukey's multiple comparison test, and differences were considered statistically significant at $P < 0.05$.

Table 1. Oligonucleotide primers used for real-time reverse-transcription PCR

RNA target[1]	Primer sequence	PCR product size (bp)	GenBank accession no.
GAPDH		264	NM_204305.1
Forward	5′-GGTGGTGCTAAGCGTGTTAT-3′		
Reverse	5′-ACCTCTGTCATCTCTCCACA-3′		
IL-1β		244	NM_204524.1
Forward	5′-TGGGCATCAAGGGCTACA-3′		
Reverse	5′-TCGGGTTGGTTGGTGATG-3′		
IL-6		254	NM_204628.1
Forward	5′-CAAGGTGACGGAGGAGGAC-3′		
Reverse	5′-TGGCGAGGAGGGATTTCT-3′		
IL-8		200	NM_205498.1
Forward	5′-GGCTTGCTAGGGGAAATGA-3′		
Reverse	5′-AGCTGACTCTGACTAGGAAACTGT-3′		
iNOS		241	U46504
Forward	5′-TGGGTGGAAGCCGAAATA-3′		
Reverse	5′-GTACCAGCCGTTGAAAGGAC-3′		
GPx7		223	NM_001163245.1
Forward	5′-TCACCACCTTCAGAATGCAG-3′		
Reverse	5′-TCCCAACTGGGAAATTCTTG-3′		

[1]GAPDH = glyceraldehyde 3-phosphate dehydrogenase; iNOS = inducible nitric oxide synthase; GPx7 = glutathione peroxidase 7.

RESULTS

Effect of In Ovo Injection with Se on BW, Intestinal Lesions, and Oocyst Production

Selenium injection did not affect overall hatchability, the hatch sex ratio, or BW at d 0 posthatch and did not produce gross pathologic effects when used at the denoted concentrations of 10 or 20 μg/egg compared with eggs injected with PBS alone (data not shown). Following coinfection with *E. maxima* and *C. perfringens*, SS0 group showed depression, diarrhea, and reduced BW compared with the uninfected control. However, chickens hatched from eggs injected with 10 (SS10) and 20 (SS20) μg of Se/egg showed less depression and diarrhea, and the SS20 group had significantly increased BW compared with the coinfected and untreated controls (**SS0**; Figure 1A). There was no significant difference in the BW of uninfected control and Se-treated and coinfected SS10 and SS20 groups. Birds treated in ovo with Se and coinfected with *E. maxima* and *C. perfringens* generally showed the trend of reduced intestinal lesion scores (Figure 1B) and the significant difference from the SS0 group was found in the SS10 group. Furthermore, chickens hatched from eggs injected with 10 (SS10) and 20 (SS20) μg of Se/egg showed decreased oocyst production (Figure 1C) compared with unsupplemented and coinfected controls.

Effect of In Ovo Injection with Se on Serum Antibody Levels to α-Toxin and NetB Toxin

Chickens in the SS10 and SS20 groups showed higher serum antibody titers against α-toxin and NetB toxin compared with the unsupplemented and coinfected SS0 group (Table 2).

Effect of In Ovo Injection with Se on Lipid Peroxidation and Antioxidant Enzyme Activities

Serum MDA concentration was significantly increased in the SS0 group coinfected with *E. maxima* and *C. perfringens* without Se supplement compared with the uninfected control group (Figure 2A). However, treatment with 10 or 20 μg/egg of Se significantly decreased MDA levels in SS10 and SS20 groups compared with the Se-untreated SS0 group. There was no significant difference in MDA levels between uninfected control and infected SS20 groups. Similarly, serum CAT enzyme activity was decreased in the Se-treated and coinfected SS10 and SS20 groups compared with the SS0 control group (Figure 2B).

Coinfection with *E. maxima* and *C. perfringens* significantly increased SOD enzyme activity in SS0 group compared with the uninfected control group (Figure 2C). However, the SS10 group showed the significantly decreased serum SOD enzyme activity compared with the SS0 control group, although there was no significant difference between SS0 and SS20 groups.

Effect of In Ovo Se Injection on Cytokine, Chemokine, and GPx7 Transcript Levels

The IL-1β and IL-6 transcript levels in the intestinal jejunum were increased in the SS20 group with Se-supplementation and *E. maxima*/*C. perfringens* coinfection, compared with the unsupplemented and coinfected SS0 control group (Figures 3A-3B). The IL-8 and iNOS transcript levels in the intestinal jejunum were increased in both of the Se-supplemented and *E. maxima*/*C. perfringens* coinfected groups (SS10, SS20) compared with the SS0 control group (Figures 3C-3D).

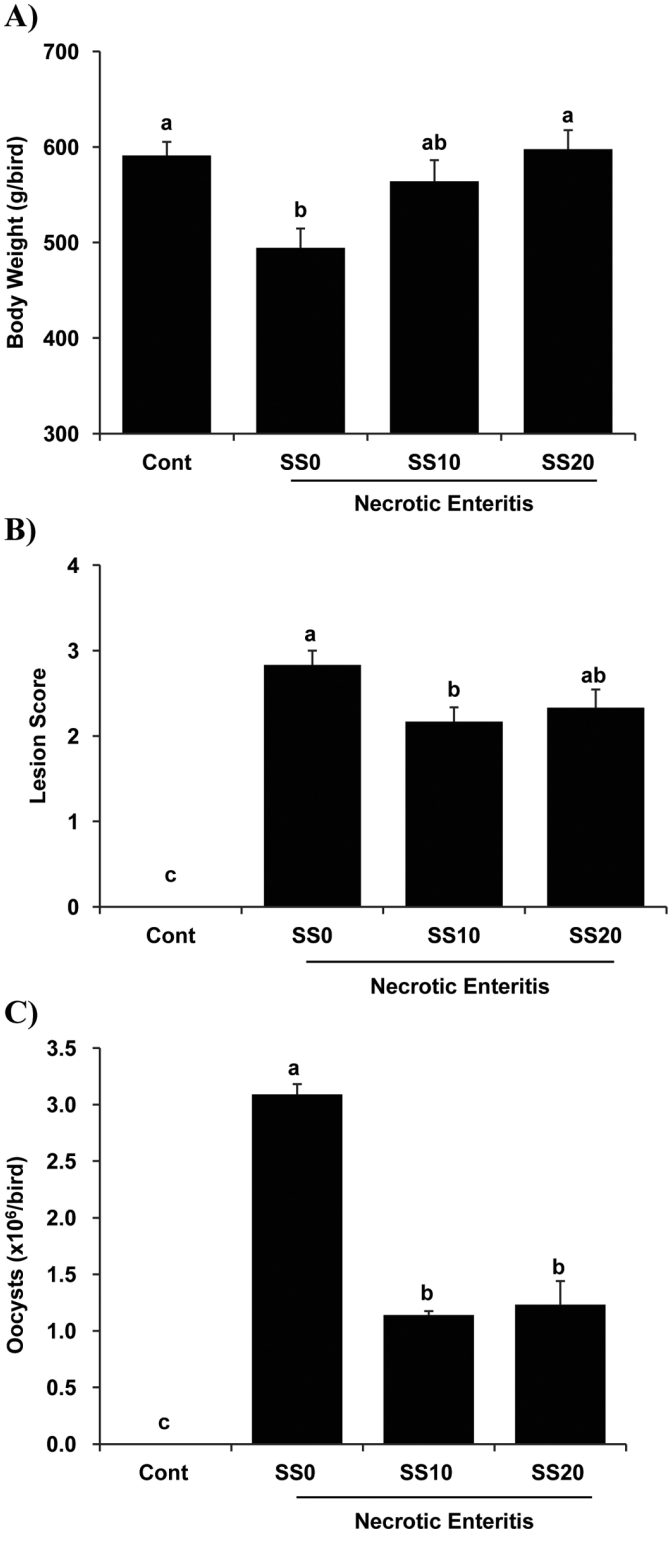

Figure 1. Effect of in ovo injection with Se on BW, intestinal lesions, and oocyst production during experimental necrotic enteritis. Broiler eggs were injected with 100 μL of PBS alone (Cont, SS0) or sodium selenite in PBS at 10 (SS10) or 20 (SS20) μg of Se/egg at 18 d of embryo age. At 14 d posthatch, chickens were uninfected (Cont) or orally infected with 1.0×10^4 sporulated oocysts of *Eimeria maxima*. At 18 d posthatch, chickens were orally infected with 1.0×10^9 cfu of *Clostridium perfringens* and the following parameters were assessed: (A) BW at d 20; (B) gut lesion scores at d 20; (C) fecal oocyst shedding between d 19 and 24 (d 5 to 10 following *E. maxima* infection). Each bar represents the mean ± SEM value (n = 20 in A; n = 5 in B; n = 15 in C). Bars not sharing the same letters (a–c) are significantly different according to Tukey's multiple comparison test at $P < 0.05$.

Higher transcript levels of IL-1β, IL-6, and iNOS seen in the intestinal jejunum of the SS20 group compared with the SS10 group suggest a Se dose-dependent effect on these cytokine genes. In contrast, intestinal GPx7 transcript levels were decreased in the Se-treated and coinfected SS10 and SS20 groups compared with the SS0 controls (Figure 3E).

Effects of In Ovo Injection with Se on Posthatch Tissue and Fecal Se Levels

Selenium levels (μg/100 g) in the thymus, heart, liver, muscle, and feces were equal in the SS0, SS10, and SS20 groups (Table 3). Interestingly, Se levels in the thymus, heart, and feces were lower in the SS10 and SS20 groups compared with the unsupplemented and uninfected controls.

DISCUSSION

This study was conducted to evaluate the effects of in ovo injection with Se on immune and antioxidant responses during experimental NE using a *Clostridium/Eimeria* coinfection model system in commercial broilers. No detrimental effects on embryo viability or hatchability were noted by either PBS or Se in ovo injection. Among the groups coinfected with *E. maxima* and *C. perfringens*, chickens hatched from eggs injected with Se showed 1) increased BW; 2) reduced gut lesions and oocyst excretion in feces; 3) decreased serum MDA levels, reduced CAT and SOD antioxidant enzyme activities, and diminished intestinal GPx7 transcript levels; and 4) improved antibody responses to α-toxin and NetB toxin and greater IL-1β, IL-6, IL-8, and iNOS transcript levels compared with the unsupplemented and coinfected chickens. These collective results suggest that in ovo Se supplementation has a protective effect against experimental NE. Increased BW, decreased intestinal lesions, and reduced oocyst production in the Se-treated groups compared with untreated controls might be attributable to improved intestinal physiology allowing for greater nutrient absorption. A similar effect was proposed previously to account for the protective effect of Se dietary supplementation in broiler chickens against infection by *E. tenella* (Colnago et al., 1984). Also consistent with the current report, Monteiro et al. (2009) and Betancor et al. (2012) observed that Se supplementation lowered MDA, CAT, and SOD activities in piscine systems.

Malondialdehyde is a reactive aldehyde, one of the many reactive electrophile species that cause toxic stress in cells and form covalent protein adducts, referred to as advanced lipoxidation end products (Farmer and Davoine, 2007). Malondialdehyde lipid peroxidation is generated from reactive oxygen species (**ROS**) and is assayed in vivo as a biomarker of oxidative stress (Moore and Roberts, 1998; Del Rio et al., 2005). The lower MDA levels in the SS10 and SS20 groups com-

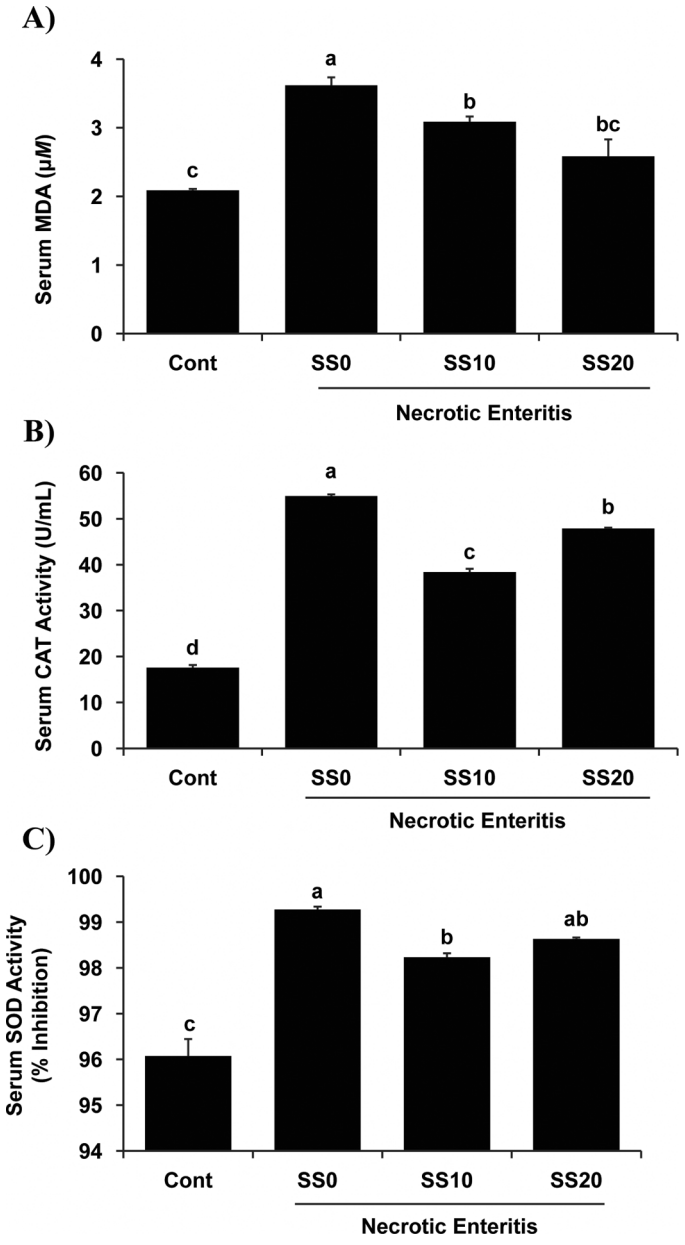

Figure 2. Effect of in ovo injection with Se on serum malondialdehyde (MDA) levels and serum catalase (CAT) and superoxide dismutase (SOD) activities during experimental necrotic enteritis. Eggs were injected with PBS alone or sodium selenite and hatched chickens were coinfected with *Eimeria maxima* and *Clostridium perfringens* as described in Figure 1. Serum MDA concentrations, and CAT and SOD activities were measured at d 20. The SOD activity is expressed as the % inhibition of WST-1 formazan formation by superoxide anion that remains following its decomposition by SOD. Each bar represents the mean ± SEM value (n = 5). Bars not sharing the same letters (a–d) are significantly different according to Tukey's multiple comparison test at $P < 0.05$.

Table 2. Effect of in ovo injection with Se on serum antibody levels to α-toxin and NetB toxin in experimental NE

	Antibody titer	
Group[1]	α-Toxin (OD_{450})[2]	NetB (OD_{450})
SS0	0.21 ± 0.02[a]	0.27 ± 0.03[a]
SS10	0.42 ± 0.03[b]	0.42 ± 0.06[b]
SS20	0.41 ± 0.02[b]	0.37 ± 0.03[b]

[a,b]Different letters within each column are significantly different according to the Tukey's multiple comparison test at $P < 0.05$.

[1]Broiler eggs were injected with 100 μL of PBS alone (SS0) or sodium selenite in PBS at 10 (SS10) or 20 (SS20) μg of Se/egg at 18 d of embryo age. At 14 d posthatch, chickens were orally infected with 1.0×10^4 sporulated oocysts of *Eimeria maxima*. At 18 d posthatch, chickens were orally infected with 1.0×10^9 cfu of *Clostridium perfringens*. Sera were collected at 6 d postinfection with *C. perfringens* and used to measure the levels of antibodies to α-toxin and NetB toxin by ELISA. Values are means ± SEM (n = 5).

[2]OD_{450} = optical density at 450 nm.

pared with the SS0 group suggest that the Se treatment groups are under reduced oxidative stress. The ROS include free radicals, such as the superoxide anion ($O_2\bullet-$), hydroxyl radical (•OH), as well as nonradical molecules such as H_2O_2 and singlet oxygen ($^1O_2$). The superoxide anion is a major component of ROS in the cell. Superoxide dismutase prevents superoxide from reacting with sensitive and critical cellular targets. Thus, SOD serves as a key antioxidant. It has been reported that the phytonutrient, *Agaricus brasiliensis* polysaccharide, inhibited lipid peroxidation and significantly lowered SOD activity in an animal model of reperfusion injury (Zhang et al., 2010). The MDA, SOD, CAT, and GPx levels in blood are lower in Se-deficient mice or chickens compared with animals with normal Se levels (Wang et al., 2009; Lee et al., 2013).

Under aerobic conditions, ROS can accumulate to toxic levels in cells. In response, the expression of antioxidative enzymes are increased to detoxify ROS. Catalase converts H_2O_2 to water and oxygen, whereas SOD catalyzes the dismutation of superoxide anion to H_2O_2 and molecular oxygen (Gaetani et al., 1996). Other relevant antioxidant enzymes include the GPx family of proteins, which includes multiple isozymes that catalyze the reduction of H_2O_2 or organic hydroperoxides to water or the corresponding alcohols using reduced glutathione as an electron donor (Fink and Scandalios, 2002). Glutathione peroxidase 7 is a monomeric glutathione peroxidase of the endoplasmic reticulum containing a cysteine redox center. Unlike other members of the GPx family, GPx7 catalyzes a peroxidatic cycle using only one cysteine residue through a mechanism in which reduced glutathione and protein disulfide isomerase serve as alternative substrates (Bosello-Travain et al., 2013).

Elaboration of proinflammatory cytokines in the avian intestinal mucosa plays a critical role in the host response to infectious pathogens (Shirley and Lillehoj, 2012). Interleukin-1β is a powerful proinflammatory cytokine capable of eliciting antibody and cell-mediated immune responses (Hong et al., 2006; Lee et al., 2010). Interleukin-8/CXCL8 (CXC ligand 8) attracts leukocytes, primarily neutrophils, to mucosal sites of inflammation. Inducible nitric oxide synthase is induced by IFN-γ (Hong et al., 2006) and high levels of iNOS transcripts were seen in the intestinal mucosa of chickens infected with *Eimeria maxima* or *Salmonella enterica* (Kim et al., 2008; Li et al., 2009). Chicken IL-1β, IL-

Table 3. Effect of in ovo injection with Se on tissue and fecal Se levels in experimental NE (dry basis, μg/100 g)

Group[1]	Thymus	Heart	Liver	Muscle	Feces
Cont	108 ± 6^{a}	188 ± 9^{a}	203 ± 9^{NS}	95 ± 5^{NS}	53 ± 3^{a}
SS0	90 ± 4^{ab}	158 ± 6^{ab}	195 ± 10^{NS}	90 ± 1^{NS}	44 ± 3^{ab}
SS10	85 ± 3^{b}	163 ± 9^{ab}	205 ± 6^{NS}	80 ± 7^{NS}	37 ± 3^{b}
SS20	85 ± 5^{b}	135 ± 13^{b}	195 ± 17^{NS}	85 ± 3^{NS}	42 ± 4^{ab}

[a,b]Different letters within each column are significantly different according to the Tukey's multiple comparison test at $P < 0.05$.

[1]Broiler eggs were injected with 100 μL of PBS alone (Cont, SS0) or sodium selenite in PBS at 10 (SS10) or 20 (SS20) μg of Se/egg at 18 d of embryo age. At 14 d posthatch, chickens were uninfected (Cont) or orally infected with 1.0×10^4 sporulated oocysts of *Eimeria maxima*. At 18 d posthatch, chickens were orally infected with 1.0×10^9 cfu of *Clostridium perfringens*. Tissues were collected at 6 d postinfection with *C. perfringens*, and Se levels were quantified using an inductively coupled plasma-mass spectrometry system. Values are means ± SEM (n = 5).

6, and IL-8 mRNA reach maximum levels in the gut between d 4 and 6 following parasite or bacterial infections to initiate an acute phase response (Swaggerty et al., 2004; Hong et al., 2006). In the current study, however, intestinal mRNA levels for IL-1β, IL-6, IL8, and iNOS showed no differences between the uninfected

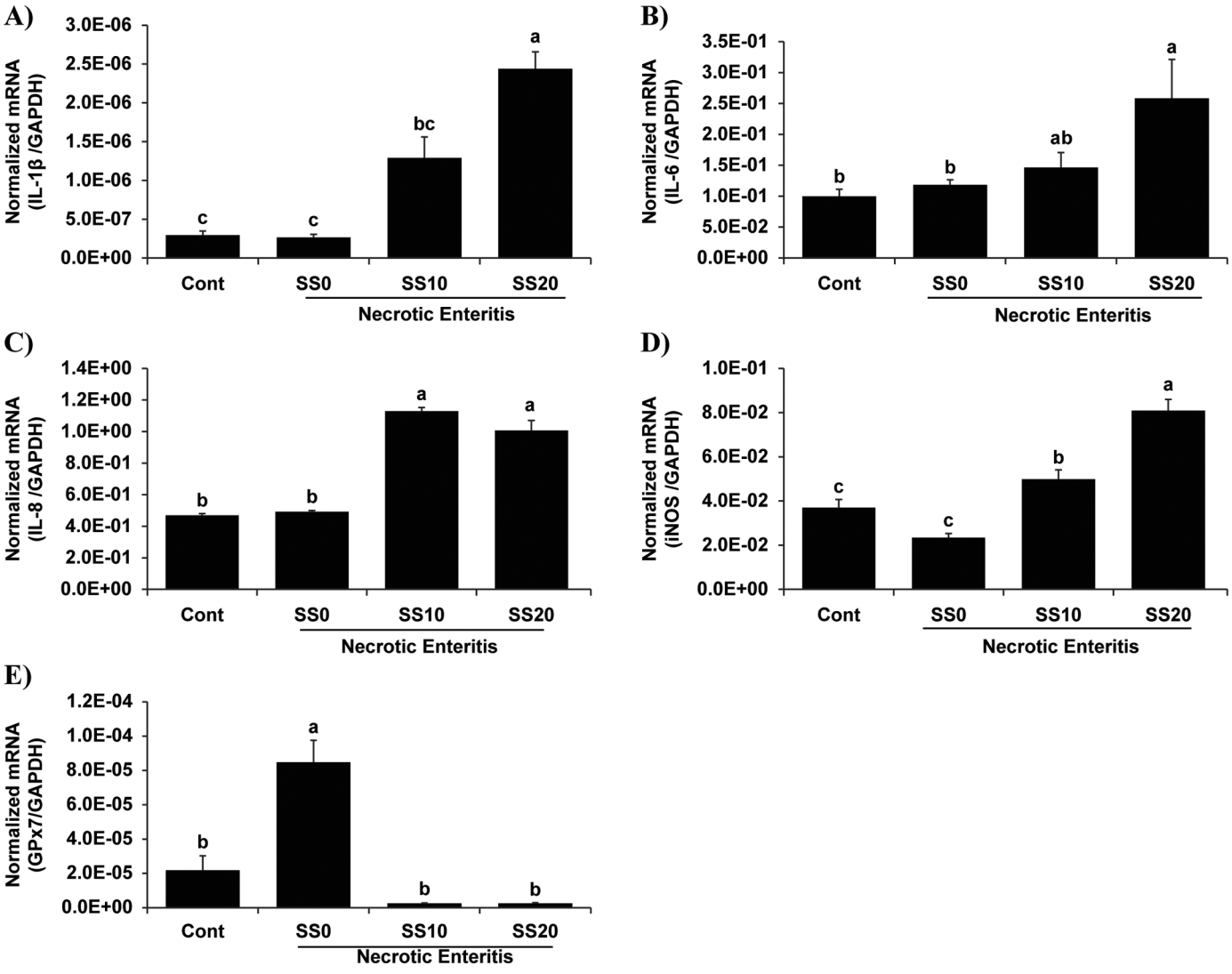

Figure 3. Effect of in ovo injection with Se on the levels of intestinal transcripts for proinflammatory cytokines, inducible nitric oxide synthase (iNOS), and glutathione peroxidase 7 (GPx7) in experimental necrotic enteritis. Eggs were injected with PBS alone or sodium selenite and hatched chickens were coinfected with *Eimeria maxima* and *Clostridium perfringens* as described in Figure 1. Intestinal epithelial cells were collected at d 20 and used to measure the levels of transcripts for IL-1β (A), IL-6 (B), IL-8 (C), iNOS (D), and GPx7 (E) by real-time reverse-transcription PCR. Individual transcript levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcript levels. Each bar represents the mean ± SEM value (n = 5). Bars not sharing the same letters (a–c) are significantly different according to Tukey's multiple comparison test at $P < 0.05$.

(Cont) and coinfected chickens treated with Se (SS0; Figure 3). This discrepancy might be explained by the different time points used in the different studies, low gene expression levels in the current study as a result of Se treatment, or both. In contrast, gut GPx7 mRNA levels were increased, as were CAT and SOD catalytic activities, in the infected SS0 group compared with the untreated and uninfected control group, indicating that *Eimeria*/*Clostridium* coinfection upregulates these critical antioxidant enzymes. That in ovo Se injection decreased GPx7 transcript levels, diminished CAT, and SOD activities, and lowered serum MDA concentrations in coinfected chickens compared with the SS0 controls suggests that this micronutrient decreases oxidative stress associated with the experimental NE model system. These results are consistent with previous studies demonstrating that dietary supplementation of chickens with Se reduced coccidiosis symptoms and altered the in vivo expression of a family of innate immune response genes compared with chickens fed an unsupplemented diet (Colnago et al., 1984).

In summary, in ovo Se treatment is an effective strategy to reduce the clinical signs of experimental avian NE and was correlated with reduced oxidative stress and increased parameters of humoral and cellular immunity. Future studies are needed to further define the molecular and cellular pathways that are affected by this micronutrient in broiler chickens.

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